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PRINCIPAL INVESTIGATOR: Nandita Sharma, Ph.D.

CONTRACTING ORGANIZATION: Stanford University
Stanford, California 94305-5401

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Nandita Sharma, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Stanford University
Stanford, California 94305-5401

E-Mail: nanditas@stanford.edu

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Identification of Estrogen-Responsive Genes and Their Role in Breast Cancer

Background

The development of breast cancer has been linked to a variety of factors, including age, parity, family history and hormonal milieu. Estrogen, the female sex hormone, is a powerful mitogen and promotes neoplastic growth in mammary epithelium. *In vitro*, estrogens have been shown to modulate human breast cell via α and β estrogen receptors (ER) (Zhou, Ng et al. 2000) While ERβ is preferentially expressed in normal breast tissue, ERα is abundantly expressed in invasive and in situ ductal carcinomas (Zhou, Ng et al. 2000). Tamoxifen, a non-steroidal anti-estrogenic drug is widely used for adjuvant therapy of breast cancer. ERα expression is currently the best method to predict if a cancer will respond to hormonal therapy. However, 35% of the primary tumors, which are ERα - positive do not respond to hormone therapy and about 10% of ERα-negative tumors are hormonally responsive (Jordan, Wolf et al. 1988). It would immensely beneficial to predict with a greater degree of accuracy how a breast tumor would respond to the treatment with tamoxifen, especially in the light of growing evidence of carcinogenic effects of tamoxifen (Fisher, Costantino et al. 1994; Phillips 2001).

Using suppression subtractive hybridization, Ghosh et al. identified a set of 14 estrogen-responsive genes that was differentially over-expressed in MCF-7 cells stimulated by beta-estradiol as compared with unstimulated cells (Ghosh, Thompson et al. 2000). Tamoxifen, repressed the expression of all these genes. PDZK1 and a novel gene - GREB1, were confirmed to be early responders to estrogen and demonstrated a significant correlation to the ER phenotype in a panel of ER positive and negative breast carcinoma cell lines. Studies in primary breast cancers showed that PDZK1 and GREB1 were overexpressed in ER-positive breast cancers as compared with ER-negative breast cancers by 19-fold and 3.5-fold respectively (Ghosh, Thompson et al. 2000). Due to the strong correlation of GREBI and PDZKI expression to estrogen related responses, these genes were chosen as markers to test drug responses in MCF7 cells and in primary breast tumors. The pattern of expression of GREB1 and PDZK1 suggests an important role for these proteins in physiological response of tumors to estrogen. There may be a trend that tumors, which exhibit tamoxifen-induced alteration in the target gene expression *in vitro*, are more likely to respond to tamoxifen therapy whereas those, which do not, may be less likely to respond to tamoxifen treatment in a clinical setting.

In order to predict a patient's response to the drug treatment more accurately, it might be more important to examine the dynamic alteration of gene expression in response to the drug treatment as opposed to merely looking for their expression at a 'static' time-point. GREB1 and PDZK1 may serve as useful markers and studying the alteration in the expression of these marker genes may be used as a tool to predict a patient's response to drug treatment.

Objectives, Methods and Results

- **Optimize the conditions to examine dynamic changes in gene expression using cancer cell lines.**

Method: The purpose of the method was to determine the minimum number of cells required, duration of drug treatment, and best method to analyze gene expression changes in breast cancer cells. Based on an initial protocol developed by M. Ghosh, the MCF7 cells were incubated in a culture medium containing stripped fetal calf sera and lacking phenol red, so as to remove all estrogens. Cells at different concentrations (5000, 10000 and 50000 cells) were treated with β-estradiol (10 nM) or β-estradiol (10 nM) and tamoxifen (1 μM) in triplicate. The cells were treated for various time-lengths viz. 1, 2, 4, 8 and 24 hours. A control group of cells, which received no drug treatment, was also analyzed for each cell density and time point. Total RNA was isolated at the end of the treatments and analyzed by RT-PCR for expression of GREB1, PDZK1, and pS2 genes. Expression level of two housekeeping genes –actin and GAPDH- was also checked by RT-PCR as a control for the total amount of RNA.

Results: The assay conditions have been successfully developed using MCF7 cells, wherein 5000 cells, which is approximately the number of tumor cells expected from a small core biopsy, are sufficient to isolate reasonable amount of RNA that can be analyzed by RT-PCR. Treatment of these cells for 4 hours shows significant changes in the expression levels of target genes upon treatment with β-estradiol and tamoxifen. Thus, it will not be necessary for the tumor cells to remain viable in the cultures for long periods of time. The number of cycles, and extension and annealing temperatures for RT-PCR of each target gene has also been optimized so as to give a signal in the linear range.

- **Develop a protocol for testing breast tumor tissue for altered gene expression upon drug treatment**

Method: The aim of this method was to determine conditions for culture and treatment of primary breast tumor tissue, since primary breast tumors are likely to behave differently than the cultured tumor cell lines. Fresh tumor specimens were collected from the primary breast cancers that were resected in the Stanford operating room. Tumor tissue was cut into small pieces, smaller than 1mm in size. This should allow for the diffusion of oxygen into the specimen. Alternatively, the minced tissue was treated with collagenase to disaggregate the tissue and isolate tumor cells. Minced tissue or disaggregated tumor cells were distributed into culture media and treated with estradiol or estradiol and tamoxifen for 4 hours as described in method 1 above. Whenever possible, multiple replicates were performed to determine intra-sample reproducibility. After the drug treatments, total RNA was isolated and expression of GREB1, PDZK1, pS2, and ERα was analyzed by RT-PCR as above. The expression levels of target genes were compared for samples with estradiol, tamoxifen, or no treatment.

Results: Ten primary breast tumor specimens were analyzed during the course of study. The first two tumor specimens were not treated with collagenase and showed poor intra-sample reproducibility. Treatment with 0.1% collagenase type III enzyme for 3-4 hours

disaggregates the tissue and the tumor cells thus obtained provide a more homogenous sample and hence better intra-sample reproducibility. The sample set (five tumor specimens) has been too small to draw any significant conclusions. However, the preliminary results obtained with these primary breast tumors indicate feasibility of the protocol to detect qualitative and semi-quantitative differences in target gene expression upon drug treatment. One of the breast tumor specimen, showed no response to tamoxifen treatment *in vitro*, even though it was ER-positive. This tumor likely falls into that category of ER-positive tumors which do not respond to hormonal therapy. While another tumor, which was ER-negative, did exhibit a decrease in the target gene expression in response to tamoxifen treatment. This tumor may possibly respond to tamoxifen treatment in a clinical setting.

The most unexpected hindrance in this study was the availability of tumors. We expected to analyze about 100 tumors in a year, but we could get only 12 tumor specimens in 7 months (October 1, 2001 till April 30 2002) Stanford Hospital. There were several reasons for this –

a) fewer than expected number of patients (only about 20 patients) consented to giving their tumor specimen for our study
b) a small number of the tumor specimens died in culture medium (for reasons unknown) making them worthless for our study
c) in some patients' who had consented to give their resected tumor for our study, the tumor was either too small or could not be located with certainty.

References

Fisher, B., J. P. Costantino, et al. (1994). "Endometrial cancer in tamoxifen-treated breast cancer patients: findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14." J. Natl. Cancer Inst. **86**: 527-537.

Ghosh, M. G., D. A. Thompson, et al. (2000). "PDZK1 and GREB1 are estrogen-regulated genes expressed in hormone-responsive breast cancer." Cancer Res. **60**: 6367-6375.

Jordan, C., M. F. Wolf, et al. (1988). Hormone Receptor Assays: Clinical Usefulness in the management of carcinoma of the breast. CRC Critical Reviews in Clin Lab Sci**:** 97-152.

Phillips, D. H. (2001). "Understanding the genotoxicity of tamoxifen." Carcinogenesis **22**(6): 839-849.

Zhou, J., S. Ng, et al. (2000). "Testosterone inhibits estrogen induced mammary epithelial proliferation and suppresses estrogen receptor expression." FASEB J. **14**: 1725-1730.

Achievements in the period 10-1-01 through 4-30-02

- Optimized the conditions such as drug dosage, time of treatment, and PCR conditions, to examine dynamic changes in gene expression.

- Developed the protocol for testing breast tumor tissue for altered gene expression upon drug treatment